Supplemental Figures

Figure S1. Compound structures. The NCBI PubChem structures and SMILES (simplified molecular input line entry specification) for multiple myeloma standard of care drugs dexamethasone, lenalidomide, bortezomib, melphalan and doxorubicin as shown. Structures and SMILES for adenosine receptor agonists CGS-21680, ADAC, Chloro-IB-MECA, HE-NECA and beta-2 adrenergic receptor agonists salmeterol, formoterol, clenbuterol, terbutaline and levalbuterol are also shown. All of the adenosine receptors agonists stimulate the A2A adenosine receptor with differences in potency and selectivity (1). The five beta-2 adrenergic receptor agonists are highly selective but differ in potency (2). Because of the properties of these agents, we have used them interchangeably throughout our work to study A2A and beta-2 receptor agonist-dependent activities.

Figure S2. Combination activity and dose-response matrix analysis. There are two useful ways in which one compound can influence the anti-disease activity of another to provide medical benefit - potency shifting and effect boosting (3). In the case of potency shifting, the magnitude of a combination effect can be evaluated by whether the addition of a second agent results in substantially less of the first agent needed to achieve the desired effect level. The evaluation of synergy in this context is particularly useful in the justification of clinically used combinations where one can define the point at which the combination provides clinical benefit that cannot be otherwise achieved by just increasing the dose of either single agent. The most commonly used model of dose additivity is Loewe additivity where the model provides a null-reference that is predicted by the expected response if the two combined agents are in fact the same drug (4). As an example consider two drugs, A and B which each individually achieve a single agent effect level of 50% at 1 uM and 2 uM respectively. The Loewe additivity model would then predict that if the combination of drug A and drug B was strictly additive, a combination of 0.5 uM of A and 1 uM of B would also give an effect level of 50%. This would be analogous to combining 0.5 uM of A to 0.5 uM of A, giving a total of 1 uM of A and achieving a 50% effect level. By quantifying the deviation from the Loewe additivity model, one can quantify the effect of the combination interaction.

One common way in which such deviations are quantified is by using the combination index (4). The combination index (CI) is the ratio of the total effective drug dose of the combination compared to that of the single agents required to achieve a given effect level. When the combination index is less than 1, less of either drug is needed to achieve the proscribed effect level than would be predicted by the Loewe additivity model and the combination can be described as synergistic. When the combination index is greater than 1, the opposite is true, i.e. more of

either drug is needed to achieve the proscribed effect level than would be predicted by Loewe additivity and the combination can be described as antagonistic.

It is important to note that a CI for any given combination describes potency shifting at a specific fixed effect level, and a unique CI must be calculated for each dose, ratio and effect level sampled. In essence, the combination index represents the numerical value associated with a single point on a 2-dimensional slice or iso-effect level through the 3-dimensional dose effect surface (in this case, two dimensions measure each single agent concentration and one measures the effect level at each dose and ratio of the two agents). This is often visualized across multiple doses and ratios of two drugs at a single effect level using an isobologram (4). In this graphical representation, pairs of doses of both compounds that achieve a fixed effect level (isoboles) are plotted along the two axes representing the concentration of each single agent where the origin in the lower left corner represents the hypothetical zero concentration of either agent. When drug concentration axes are normalized to the concentration at which the single agent achieves the proscribed effect level, the isobols become symmetric with a straight line predicting concentrations for additivity extending diagonally between the two single agent effect levels achieving the proscribed effect. A contour drawn through the pairs of doses achieving the specified effect is used to visualize the deviation of the combination from the additivity line. The isobologram provides a simple and straightforward way to evaluate all combination index data across a single effect level.

Because combination interactions can occur over a wide range of different doses and ratios it is important to comprehensively sample the entire 3-dimensional dose effect surface. To do this, one must look at all possible effect levels achieved by all doses and ratios of the two single agents, in essence visualizing the 3-dimensional surface of the combination. We have approached this problem by evaluating all possible concentrations and ratios of two single agents in a full dose-matrix design (5,6). In this context, using a null-effect reference model such as Loewe additivity allows the calculation of a 3- dimensional model surface predicted by the single agent response curves. This surface can then be compared to the 3-dimensional dose effect surface empirically derived from tests of all doses and ratios of each of the single agents in combination. Simply subtracting the model surface predicted from the null-effect model from the observed, empirically derived surface allows the calculation of a difference volume representing deviation from the model.

This method has the advantage of emphasizing the combination interaction across a broad range of concentrations and ratios, minimizing the effects of individual outliers and facilitates the identification of combinations with robust combination effects. Furthermore, by using a set of shape models to characterize the

morphology of the full response surfaces one can infer a relationship between the dose matrix response shape of a combination and the connectivity of the targets engaged by the combination (5). An example of the dose matrix design for combination analysis and some experimental results are provided:

Dose-Response Matrix Analysis. 2A) Using the cHTS platform, two agents can be screened in combination using a format such as a 6x6 dose-response matrix (left matrix) to capture a wide range of concentrations and ratios. Higher activity levels are displayed using a brighter/warmer matrix of colors. Using the data collected for the two single agents, combination data was modeled for a null interaction between the components of the combination (middle matrix, Loewe Additivity model). Loewe dose additivity is the expected response if both agents inhibit the same molecular target by the same mechanism. This null interaction matrix is subtracted from the experimentally-derived data (left matrix, the data surface) to identify activity values in excess (right matrix) of what would be expected if there is no interaction between the components. This excess matrix volume can be integrated to generate a synergy score. The synergy score equation: $S = \ln fX \ln fY \Sigma\text{doses } \max(0, Z\text{data}) (Z\text{data}-Z\text{Loewe})$ integrates the experimentally- observed activity volume at each point in the matrix in excess of a model surface numerically derived from the activity of the component agents using the Loewe model for Additivity. Additional terms in the synergy score equation normalize for various dilution factors used for individual agents, bias for synergistic interactions that occur at high activity levels and allow for comparison of synergy scores across an entire experiment. **B and C)** Generation of Additivity excess matrix volumes for the CGS-21680 x Dex and Salmeterol x Dex matrices shown in Figure 1. The synergy scores for these combinations are 6.58 and 8.33 respectively. **2B)** Dose matrix analysis of adenosine receptor agonists in combination with dexamethasone. Dexamethasone was crossed with the adenosine receptor agonists HE-NECA, Chloro-IB-MECA and ADAC in MM.1S cells using a 9x9 dose matrix combination (HE-NECA and Chloro-IB-MECA) or 6x6 (ADAC) screening format. The HE-NECA data is the average of two matrices Chloro-IB-MECA four and ADAC the average for three matrices. Activity in excess of additivity has been plotted across the matrices (middle panels) with activity provided numerically and by color. The isobologram analysis is on the right. **2C)** Dose matrix analysis of beta 2 receptor agonists in combination with dexamethasone. The same as in S1B except that Dex was crossed with terbutaline, formoterol and clenbuterol, with all crosses in the 9x9 format. The terbutaline and formoterol

crosses were performed in duplicate while the clenbuterol crosses were done in quadruplicate.

Figure S3. Analysis of AdR and β2AR agonist combinations with dexamethasone in additional multiple myeloma cell lines. A) CGS-21680 was crossed with Dex in a 9x9 dose matrix format in RPMI-8226, OPM-2, ANBL-6, EJM, H929 and MM.1R cell lines. Activity in excess of additivity has been plotted across the matrices with activity provided numerically and by color. **B)** Same analysis as in (A) except with salmeterol and Dex.

Figure S4. Analysis of AdR and β2AR agonist combination activity with multiple myeloma standard of care drugs lenalidomide, melphalan, doxorubicin and bortezomib. The AdR agonist was CGS-21680 **(A)**, the β2AR agonist was salmeterol **(B)**. 9x9 dose matrix analysis was performed using the multiple myeloma cell line MM.1S with both inhibition and additivity excess volume displayed.

Figure S5. The combination of adenosine or beta-2 adrenergic receptor agonists with dexamethasone, lenalidomide or bortezomib potently induces multiple myeloma cell death. A) MM.1S cells were incubated with 20nM CGS-21680 (AdR), 130pM salmeterol (β2AR), 20nM Dex or drug combinations at 4x 10^5 cells/ ml in a six-well tissue culture dish for 24 or 48 hrs, cells were harvested and analyzed by FACS for Annexin V positive cells. Apoptosis analysis was performed using the Annexin-V-Fluos Staining Kit according to the manufacturer's instructions (BD Biosciences), followed by flow cytometric analysis with a BD FACS Caliber. FACS plots were generated and percent Annexin V positive cells determined. The experiment was performed 3 times and data from one is shown. **B)** Amount of MM.1S cell apoptosis induced after 96 hr exposure to 50nM CGS-21680 (AdR), 30pM salmeterol ((β2AR), 1uM lenalidomide (Len) and drug combinations. **C)** MM.1S apoptosis induced by 72 hr exposure to 50nM GCS-21680 (AdR), 300pM salmeterol ((β2AR), 2nM bortezomib (Bort) and drug combination. **D)** Colony forming assays- the number of RPMI-8226 colonies growing in Methocult H4320 after 5 hours exposure to 100nM CGS 21680 (AdR), 2nM salmeterol ((β2AR), 100nM bortezomib, 200nM bortezomib or combinations. Bortezomib as a single agent has a steep dose response curve resulting in a quick transition from cytostatic to cytotoxic effects with assay of 2-fold dilutions of drug (see Figure S4A,B). Colony forming assays were used to

evaluate the effects of AdR or β2AR agonists in combination with cytotoxic concentrations of bortezomib, as large numbers of cells can be treated with single agents or drug combinations with the assay providing a good measure of survival for the remaining few cells. We were unable to use MM.1S cells for the studies, as we could not optimize conditions for colony formation which is assumed to be due to lack of cell growth at the low cell plating densities. After drug treatment, cells were washed twice with PBS and plated on methylcellulose culture (MethoCult H4320, Stem Cell Technology, Vancouver, BC, Canada) at $1x10^3$, $4x10^3$ and $1.6x10^4$ cells/ plate in duplicates. After two week incubation at 37oC, colonies were counted. Only the plates whose colony count was between 25 and 250 were scored.

Figure S6. Chemical and molecular genetic validation of the beta-2 adrenergic receptor subtype as a novel multiple myeloma drug targets through use of β2AR antagonists butoxamine, ICI-118,551 and β2AR siRNA. A) Clenbuterol was crossed with lenalidomide in MM.1S cells using a 6x6 dose matrix format in the absence or presence of 0.9uM or 9uM of the β2AR antagonist butoxamine (7). After addition of compounds, cells were incubated for 72 hours prior to the addition of ATP lite. Similar results are obtained when Dex is used instead of lenalidomide. Butoxamine reduces clenbuterol and Dex/clenbuterol activity but does not affect Dex single agent activity. **B)** Information about inhibition when cells were incubated with 0.25uM lenalidomide, 0.005uM clenbuterol, or both drugs in the absence or presence of 0.9 uM or 9uM of butoxamine was taken from the dose matrix and presented as a bar graph to highlight that butoxamine effects are specific to clenbuterol. **C)** Clenbuterol was crossed with Dex in the absence or presence of β2AR antagonist 9nM ICI 118,551. D) **B)** MM.1R cells were electroporated with 50nM of the non-selective siRNA siCON (control), or siRNAs targeting the beta-2 adrenergic receptor or the A2A subtype adenosine receptor. Forty eight hours later, 0.1uM levalbuterol was added to the cultures and cells incubated an additional 72 hours prior to addition of ATP lite, with each measurement performed in triplicate. As determined by RT-PCR, at the time of drug addition, target RNA knockdown was 78% for the A2A receptor siRNA and 34% and 60% for the beta 2 adrenergic receptor siRNAs β2AR -1 (ADRB2-s1123) and β2AR -2 (ADRB2-s229200) respectively. siRNA were purchased from

Dharmacon (siCON) and Applied Biosystems (ADRB2-s1123 and ADRB2-s229200). Electroporation of siRNA was performed as described (8).

Figure S7. 9x9 dose matrix screen of 83 tumor cell lines to determine selectivity and breadth of activity of A2AR and β2AR agonists in combination with melphalan. A 9x9 dose matrix was generated as described in Figure 1. **A)** The starting concentration for CGS-21680 was 0.2uM, melphalan was 21uM. Each was diluted 2 fold. **B)** The starting concentration for salmeterol was 5nM (2 fold dilutions) and 21uM for melphalan (2 fold dilutions). There were 2-4 replicates for each assay. To assess synergy, each test point is compared to the dose-additive model (expectation for a drug crossed with itself) that is calculated at every test point in the matrix using the single agent responses at the edges. See Supplemental Figure 1A for additional details. BCM = non-MM B cell malignancies.

Figure S8. The A2A AdR agonist CGS-21680 and β2AR agonist salmeterol do not synergize with the multiple myeloma standard of care agents Dex or bortezomib in non-transformed cells. The single agent dose response for CGS-21680 and salmeterol was determined after 72 hour incubation in MM.1S (panels A, C and D), H929 (panel B), peripheral blood mononuclear cells (PBMC, panels A, B and D), human coronary artery endothelial cells (HCAEC, panel C).The dose response was also determined when 75nM Dex was added (panel A and C), or after addition of 2.5nM bortezomib (panels B and D). Percent inhibition was determined using ATP lite

Figure S9. Microarray analysis of genes coordinately upregulated or downregulated upon combination drug treatment. Affymetrix U133 plus 2.0 cDNA microarrays was used to investigate the differentially expressed genes in a multiple myeloma MM1.S cell line either untreated or treated for six hours with CGS 21680 (CGS, 12.5 nM), Salmeterol (Sal, 1nM), low dose dexamethasone (Dex, 25nM), high dose dexamethasone (highDex, 2μM), or the combination of low dose Dex plus CGS-21680 or Salmeterol (CGS+Dex, Sal-Dex). The data were normalized, processed and filtered with dChip software (standard deviation across logged data > 0.45). Unsupervised hierarchical clustering analyses showed

two major clusters with genes highly up-regulated or down-regulated in the combination groups, suggesting a unique mechanism underlying the combination treatments. With statistical analysis of microarray (SAM) analysis, we found 314 and 309 genes that showed statistically significant up-regulation or down-regulation in the combination groups compared to the untreated control, with FDR =< 1% (data not shown). It is noteworthy that only a few genes were statistically different between CGS with Dex and Sal with Dex (data not shown), suggesting that they may function with a similar mechanism. However, we do note that the combination of CGS-21680 and Salmeterol has synergistic antiproliferative activity in MM.1S cells, so although they share similar mechanisms of action, each agent also has distinct effects.

Figure S10. Gene Set Enrichment Analysis (GSEA). A) GSEA enrichment plot. Transcription factor IRF4 target genes (9) are differentially regulated in MM1.S cell line treated with the combination of low dose dexamthasone (25nM) plus CGS-21680 (12.5nM) or Salmeterol (1nM) (CGS + Dex , Sal + Dex) as compared to the single agents CGS 21680, Salmeterol, low dose dexamethasone, or high dose dexamethasone (highDex, 2μM). GSEA (10,11) was used to determine if combination treatments are enriched with IRF4 and genes regulated by IRF4. The affymetrix U133plus2 gene expression dataset obtained from the aforementioned samples were used to query IRF4 genesets in the GSEA database. The top ranked list of the differential expressed genes between the combination treated vs. untreated or single agent treated samples (color bar) are highly enriched with direct targets of IRF4 in multiple myeloma geneset (Shaffer_IRF4_Multiple_Myeloma_Program) (vertical black lines) . The normalized enrichment score (NES) is 1.34 (qval = 0.12). A number of the IRF4 target genes listed in Fig.5 (such as MYC, PIM2, SCD, CDK6 and SUB1) are among the genes at the leading edge of GSEA, which contribute significantly to the enrichment scores. **B) Heatmap of IRF4 target genes expression changes after drug treatment.** A heatmap of the IRF4 geneset (Shaffer_IRF4_Multiple_Myeloma_Program) is shown. The gene expression values in the untreated or single agent treated MM1.S cells (grey) vs. the combination treated (highlighted in yellow) are presented, where the range of colors (red, pink, light blue, dark blue) shows the range of their relative expressions (high, moderate, low and lowest, respectively). The genes are sorted according to correlation between the two groups (untreated and single agents vs. combinations).

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